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In vitro Inhibition of Human CYP1A2, CYP2D6, and CYP3A4 by Six Herbs Commonly Used in Pregnancy

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Black elderberry, cranberry, fennel, ginger, horsetail, and raspberry leaf, herbs frequently used in pregnancy, were investigated for their *in vitro* CYP1A2, 2D6, and 3A4 inhibitory potential. Aqueous or ethanolic extracts were made from commercially available herbal products, and incubations were performed with recombinant cDNA-expressed human CYP enzymes in the presence of positive inhibitory controls. Metabolite formation was determined by validated LCMS/MS or HPLC methodologies. IC_{50} inhibition constants were estimated from CYP activity inhibition plots using non-linear regression. The most potent inhibition was shown for fennel towards CYP2D6 and 3A4 with respective IC_{50} constants of 23 ± 2 and 40 ± 4 μg/ml, horsetail towards CYP1A2 with an IC_{50} constant of 27 ± 1 μg/ml, and raspberry leaf towards CYP1A2, 2D6, and 3A4 with IC_{50} constants of 44 ± 2, 47 ± 8, and 81 ± 11 μg/ml, respectively. Based on the recommended dosing of the different commercial herbal products, clinically relevant systemic CYP inhibitions could be possible for fennel, horsetail, and raspberry leaf. In addition, fennel and raspberry leaf might cause a clinically relevant inhibition of intestinal CYP3A4. The *in vivo* inhibitory potential of these herbs towards specific CYP enzymes should be further investigated. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: cytochrome P450; CYP inhibition; *in vitro*; herbal product; pregnancy; herb-drug interactions.

INTRODUCTION

The last decades have shown an increase in consumption of herbal products in the Western society, for women in particular (Messerer *et al.*, 2001; Sibbritt *et al.*, 2011). Contributing to this is the escalating appeal to nature, a common fallacy that products derived from natural sources must be safe and without side effects. About one-third of conventional drugs are, however, developed from plants, for instance digitalis and morphine, demonstrating the great potential for herbs or herbal extracts being pharmacologically active (Singh *et al.*, 2012). As for conventional drugs, active constituents in herbal preparations may also cause serious side effects and drug interactions. Nevertheless, manufacturers of herbal products are generally not required to provide documentation of safety, efficacy, nor quality of their products, unless they are registered and marketed as products with a specific proven medical claim.

The increasing consumption of herbal products allows for more unforeseen herb–drug interactions. The underlying pharmacological mechanisms for most reported herb–drug interactions are not fully understood (Chen *et al.*, 2012). When administered simultaneously, herbs may alter the drug concentration by affecting metabolizing enzymes in the cytochrome P450 (CYP) superfamily, which may lead to therapeutic failure or unwanted side effects. Many reported drug interactions caused by herbal intake have shown limited clinical consequences (Izzo, 2012). However, some herb–drug interactions may have caused severe clinical outcomes like apraxia, mania, transplant rejection, coma, or death, which is suggested for ginkgo, ginseng, kava, and St. John's wort when co-administrated, respectively, with commonly used drugs as anticoagulants, antidepressants, sedatives, or immunosuppressants (Posadzki *et al.*, 2012).

Despite the lack of documentation of content (Garrard *et al.*, 2003), efficacy (Dante *et al.*, 2012), adverse effects, and drug interactions (Singh *et al.*, 2012), herbal products are extensively used for treating pregnancy-related problems, even though this group in particular is requested to use such products with caution. It is estimated that one-third of pregnant women consume herbal products (Nordeng and Havnen, 2004; Hall *et al.*, 2011), including some that are potentially harmful (Cuzzolin *et al.*, 2010). As for herbal consumption, there is great variation in both extent and content of drug use in pregnancy. A common belief is that it is advantageous for the offspring to avoid medications; nevertheless, it is estimated that 80% of pregnant women use drugs (Headley *et al.*, 2004; Nordeng *et al.*, 2010). Relevant drugs comprise treatments for co-existing conditions as well as treatments specifically for pregnancy-related problems, and prior drug use has shown to be an important predicting factor for

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usage of both prescribed and over-the-counter drugs during pregnancy (Odalovic *et al*., 2013). By postponing pregnancies to a later age, more women also have chronic illnesses which necessitate medications for both foetal and maternal health. Concurrent usage of conventional drugs and herbal products is therefore widespread (Wade *et al*., 2008), and due to the assumed safety of natural products, patients tend not to disclose the herbal consumption to their physicians (Hall *et al*., 2011).

CYP1A2, 2D6, and 3A4 are enzymes of the CYP superfamily responsible for 65–80% of all CYP mediated drug metabolism (Ingelman-Sundberg, 2004). Examples of drugs listed as frequently used during pregnancy which principally are metabolized by these enzymes are amitriptyline, codeine, erythromycin, fluoxetine, metoclopramide, nifedipine, olanzapine, and promethazine (Glover *et al*., 2003; Tracy *et al*., 2005), indicating possible metabolic interactions when used concomitant with herbal products with a significant potential of inhibiting these enzymes. Also of interest, but not covered in this paper, are CYP2C9 and 2C19 which account for 15% of all CYP mediated drug metabolism (Ingelman-Sundberg, 2004).

Several herbs, consumed for pregnancy-related conditions, are not sufficiently investigated regarding possible CYP inhibitory potential. Among the most frequently used herbs during pregnancy, we found black elderberry (*Sambucus nigra*), cranberry (*Vaccinium macrocarpon*), fennel (*Foeniculum vulgare*), ginger (*Zingiber officinale*), horsetail (*Equisetum arvense*) and raspberry leaf (*Rubus idaeus*); herbs traditionally used for preventing influenza, urinary tract infections, digestive problems, nausea, oedema, and for promoting easier labour, respectively (Nordeng and Havnen, 2004; Cuzzolin *et al*., 2010). The objectives of this study were to determine and discuss the *in vitro* potential of the six above-mentioned herbs to inhibit human CYP1A2, CYP2D6, and CYP3A4 activities, by assessing individual IC_{50} inhibition constants, inhibition profiles, and theoretical possibilities of *in vivo* inhibition.

MATERIALS AND METHODS

Enzymes. cDNA baculovirus-expressed human CYP1A2 supersomes® (cat. no. 456203, lot no. 80669), CYP2D6 supersomes® (cat. no. 456217, lot no. 38273), CYP3A4 supersomes® (cat. no. 456202, lot no. 38275, also containing cytochrome b5), and NADPH regenerating system Solution A (31 mM NADP+, 66 mM glucose-6-phosphate, 66 mM MgCl2 in H2O, cat. no. 451220, lot nos 67359 and 79798) and Solution B (40 U/mM glucose-6-phosphate dehydrogenase in 5 mM sodium citrate, cat. no. 451200, lot nos 56568 and 82584) were all obtained from BD Gentest (Woburn, MA, USA).

Chemicals. Acetaminophen (lot no. 116K0124), ammonium acetate (lot no. 0001432367), caffeine (lot no. 0001400932), dextromethorphan (lot no. 013K1428), dextrorphan (lot no. 085K4631), 6β-OH-testosterone (lot no. 22406011), ketoconazole (lot no. 121H0524), β-naphthoflavone (lot no. 85H0356), phenacetin (lot no. S4187644207B11), quinidine (lot no. 1295350), sodium carbonate (lot no. 049K0086), testosterone (lot no. 1166233), formic acid, and hydrochloric acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). KH_2PO_4 and K_2HPO_4 were purchased from Merck (Darmstadt, Germany), acetonitrile, methanol, and toluene from Lab-Scan Analytical Sciences (Gliwice, Poland), and ethanol from Arcus Kjemi (Norway). All chemicals were of p.a. grade.

Herbal products. Cranberry tablets (Weifa Complete®, batch no. 0831641), ginger capsules (Gevita, batch no. 010308), and horsetail tea (Apotekproduksjon, lot no. 09B083/7) were bought at a retail pharmacy, and black elderberry tablets (SambuActin™, Solaray®, lot no. 121107, also containing vitamin C), fennel tea (KlosterUrter, batch no. 520900801), and raspberry leaf capsules (Nature's Sunshine, lot no. 00175479) were purchased from local health food stores.

Preparation of herbal extracts. In each of the three different CYP inhibition assays, the content of ginger and raspberry leaf capsules were ejected, and black elderberry and cranberry tablets were crushed in a mortar. The different capsule contents and crushed tablets were weighed, ranging from 360 to 1330 mg, before each being dissolved in 15 ml 60% ethanol. The solutions were heated at 30 °C with constant stirring for 60 min, centrifuged at 3000 rpm (1600 × *g*) for 10 min at room temperature, and decanted in a pre-weighed tube. The remaining pellets were re-dissolved in 5 ml 60% ethanol in the same manner and decanted into the same tubes. The fennel and horsetail teas were prepared as proposed by the manufacturers. Roughly 800 mg of fennel was crushed in a mortar, weighed, and steeped in 40 ml boiled purified water for 10 min. The same amount of horsetail was weighed and boiled directly in 40 ml purified water for 20 min. Both teas were centrifuged at 3000 rpm (1600 × *g*) for 10 min at room temperature and decanted into pre-weighed tubes. After evaporation of all six solutions at 37 °C, the dry herbal extracts were weighed. The black elderberry extract could not be fully evaporated, holding 103% of its pre-extraction weight. It was therefore resolved in 1000 µl 60% ethanol, giving a total volume of approximately 1670 µl 36% ethanol, and the herbal concentration was calculated from the weighed difference between the herbal solvent and pure 36% ethanol. Herbal stock solutions of known concentrations for the other herbs were made with 60% ethanol, except for 4% ethanol for the teas, using as little solvent as possible and if necessary using ultrasound to facilitate dissolution. All stock solutions were kept at 4 °C, protected from light and used within 1 week. 4% ethanol working solutions were made each experimental day from the stock solutions by addition of 0.1 M potassium phosphate buffer (pH 7.40).

Incubation assays. Herbal solution (100 µl, seven different concentrations for each herb ranging from 4 to 28000 µg/ml in 4% ethanol) or positive inhibition control (20 µl 1.2 µM β-naphthoflavone, 100 µl 0.2 µM quinidine, or 100 µl 1.4 µM ketoconazole for CYP1A2, 2D6, and 3A4, respectively) was added to series of three parallel conical glass tubes, together with 20 µl 8 U/ml NADPH regenerating Solution B, substrate (100 µl 0.6 mM phenacetin in 1.2% ethanol, 32 µl 0.1 mM dextromethorphan, or 50 µl 0.8 mM testosterone in 7.8%

acetonitrile), CYP enzyme (160 µl 50 nM CYP1A2, 80 µl 50 nM 2D6, or 160 µl 50 nM 3A4), and 0.1 M potassium phosphate buffer (pH 7.40). The content of organic solvents was necessary for dissolving the listed compounds, and because organic solvents affect the enzyme reactions (Busby *et al*., 1999), it was ensured that control (reference) and herbal incubations always contained the same amount and as little as possible of the solvents in question. CYP1A2 incubations were performed in 1.3% ethanol, except for the positive inhibition control which contained 0.3% ethanol and 0.5% acetonitrile, CYP2D6 incubations in 1% ethanol, and CYP3A4 incubations in 1% ethanol and 1% acetonitrile. The samples were placed in a 37 °C shaking water bath. After a 5 min preincubation period, CYP metabolism was initiated by addition of 20 µl NADPH regenerating Solution A, giving a total incubation volume of 400 µl. The incubations were terminated on ice and by addition of a 200 µl ice cold stop solution (70% acetonitrile, 10% formic acid, and 5 mM ammonium acetate for CYP1A2, 100% acetonitrile for CYP2D6, and 100% methanol for CYP3A4 assays, after 25, 25, and 10 min, respectively). CYP1A2 and 3A4 incubation samples were centrifuged at 3000 rpm (1600 × *g*) for 10 min, and the supernatants were transferred directly to high performance liquid chromatographic (HPLC) vials. Dextrorphan was extracted from the CYP2D6 incubation samples with sodium carbonate, toluene, and hydrochloric acid as described by Hellum and Nilsen (2007) and evaporated to dryness under nitrogen. The pellet was re-suspended in 500 µl 0.01 M potassium-phosphate buffer (pH 3.4) and transferred to HPLC vials.

LCMS/MS determination of acetaminophen. The CYP1A2 metabolite, acetaminophen, was determined with a liquid chromatographic tandem mass spectrometric (LCMS/MS) system consisting of a Shimadzu Prominence UFLCXR with an Eclipse XDB-C_{18} column (5 µm, 4.6 × 150 mm) interfaced to an AB SCIEX Triple Quad™ 5500 with an electrospray ion source. A gradient was generated with mobile phase A, 0.1% formic acid, and mobile phase B, acetonitrile containing 0.1% formic acid, by gradually increasing mobile phase B from 25 to 70% and flow rate from 0.8 to 1 ml/min over the first 4 min before returning to the starting conditions at 4 min. Total run time was 5.3 min, and the injection volume was 5 µl. Mass spectrometric data was acquired in positive ion mode with the following parameters: IonSpray voltage: 5000 V, temperature: 590 °C, curtain gas: 15, ion source gas 1: 75, and ion source gas 2: 55. Caffeine (775 nM) was used as internal standard. Retention time and mass transitions (m/z) for acetaminophen were 2.4 min and 152–110, and for caffeine 2.5 min and 195–138, respectively. Lower limit of quantification was 50 nM.

HPLC determination of dextrorphan and 6β-hydroxy-testosterone. The CYP2D6 and CYP3A4 metabolites, dextrorphan and 6β-hydroxy-testosterone, were determined with a HPLC system consisting of an Agilent Technologies 1200 Series with an Eclipse XDB-C_{18} column (5 µm, 4.6 × 150 mm). For determination of dextrorphan, a gradient was generated with mobile phase A, 0.01 M potassium phosphate buffer (pH 3.4), and mobile phase B, acetonitrile, increasing mobile phase B from 20 to 40% after 5 min before returning to the starting conditions at 10 min. Flow rate was 1 ml/min, and total run time was 15 min. The injection volume was 30 µl. Peak areas were registered by fluorescence detection at 230 nm excitation and 330 nm emission. Retention time of dextrorphan was approximately 4.2 min, and lower limit of quantification was 10 nM. For determination of 6β-hydroxy-testosterone, a gradient was generated with mobile phase A, H_2O, and mobile phase B, methanol. Mobile phase B was increased from 50 to 80% after 7.3 min before returning to starting conditions at 11 min. Flow rate was 1 ml/min, and total run time was 15 min. The injection volume was 40 µl. Peak areas were registered by ultraviolet detection at 240 nm. Retention time of 6β-hydroxy-testosterone was approximately 7.8 min, and lower limit of quantification was 62.5 nM.

Validation of incubation assays and metabolite determination. Mean CYP activity for the positive inhibitory controls in the incubation assays, 0.06 µM β-naphthoflavone for CYP1A2, 0.05 µM quinidine for CYP2D6, and 0.35 µM ketoconazole for 3A4, was 65 ± 9, 52 ±7, and 30 ± 3% of the control (reference) activities, respectively. Inter-run variation for the positive inhibitory controls and the control activities were in accordance with the pre-set acceptance criteria of CV < 15% for all incubation assays. The extraction recovery for dextrorphan was within 85.2–105.5% and did not differ significantly for low and high concentrations (n = 7).

Pre-run validation for determination of acetaminophen showed intraday CVs less than 8.5%, 5.3%, and 10.3% for low (200 nM), medium (2000 nM), and high (4000 nM) concentrations, respectively (n = 5), while interday CVs for the same concentrations were 5.5%, 4.3%, and 6.7%, respectively (n = 15). All quality controls during the pre-run validation had an accuracy within 98.2–103.5% (n = 45). Lower limit of quantification was 50 nM (CV 7.1%, n = 15). Pre-run validation for determination of dextrorphan and 6β-hydroxy-testosterone has previously been published by Hellum and Nilsen (2007; 2008). The pre-run validation acceptance criteria from the U.S. Food and Drug Administration (FDA) guidelines (2001) were fulfilled.

For in-run validation, standard curves with quadratic regression and 1/X weighting were constructed for acetaminophen from 50–6000 nM (7 points, $R^2 \geq 0.995$) with quality controls in duplicate at 200, 2000, and 4000 nM. Standard curves with linear regression were constructed for dextrorphan from 10 to 3000 nM (7 points, $R^2 \geq 0.9999$) with quality controls in duplicate at 30, 300, and 2000 nM and for 6β-hydroxy-testosterone from 62.5 to 20000 nM (7 points, $R^2 \geq 0.9999$) with quality controls in duplicate at 200, 3600, and 8000 nM. All quality controls deviated <15% from their nominal values throughout all trials and fulfilled the acceptance criteria for analytical series from the FDA guidelines (2001).

Calculation and statistics. Enzyme activity was expressed as pmol metabolite formed per pmol CYP and incubation time in minutes. The amount of metabolite formed was determined from area under the curves (AUCs) and standard curves of known concentrations. For LCMS/MS measurements of acetaminophen, data acquisition, peak

integration, and calculation were assigned to Analyst® software 1.5.1. For HPLC measurements of dextrorphan and 6β-hydroxy-testosterone, AUCs were manually integrated in Agilent ChemStation software. Mean % CYP enzyme activities ± SD of triplicate inhibitor incubations relative to control incubations, which were considered as having 100% enzyme activity, were plotted in SigmaPlot version 11.1.0. Half maximal and 25% inhibition concentrations (IC_{50} and IC_{25}) ± SD were estimated from the CYP activity inhibition plots by using the best fitted equation of $y = y0 + a*exp(-b*x)$ or $y = min + (max-min)/(1 + (x/EC50)^{\wedge}(-Hillslope)$. Statistical analyses were performed on Microsoft Excel 2010 (Microsoft Cooperation, Redmond, WA, USA). A paired *t*-test was applied for differences in IC_{50} constants and IC_{50}/IC_{25} ratios and 95% confidence intervals (CI) were estimated for the IC_{50} constants. A p-value of <0.05 or non-overlapping 95% CI was set *a priori* to be statistically significant.

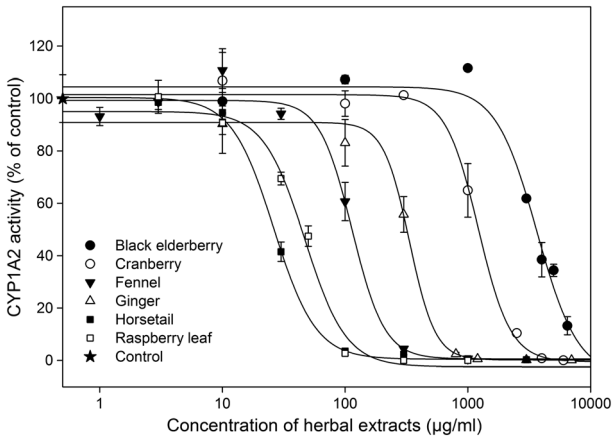

Figure 1. *In vitro* inhibition of CYP1A2 activity by the different herbal extracts. Experimental points represent mean values ± SD of triplicate incubations. The control samples without herbal extracts represent 100% enzyme activity. All curves fit the experimental points with $R^2 \geq 0.981$ ($p < 0.05$).

RESULTS

CYP1A2, 2D6, and 3A4 activity inhibition plots for the different herbal products, black elderberry, cranberry, fennel, ginger, horsetail, and raspberry leaf, are shown in Figures 1–3. All 18 inhibition plots had significant fits to the experimental points ($R^2 \geq 0.981$, $p < 0.05$). The highest concentration for each herb inhibited all three enzymes by 100%, except for black elderberry, for which such high concentration could not be achieved because of insolubility. In its highest concentration, this herb inhibited all enzymes more than 50% and thereby exceeded its IC_{50} concentrations.

Table 1 shows the IC_{50} constants of each herb for all CYP enzymes investigated and which daily dosages the manufacturers recommend for their herbal product. Because tea leaves are not ingested, dosages for fennel and horsetail tea are given as dry weight recoveries from the aqueous extraction of one dose; 9% of 3945 mg and 20% of 2430 mg, respectively. Dosing listed for black elderberry, cranberry, ginger, and raspberry leaf represents the dry weight herbal content of one tablet or capsule as claimed by the manufacturer. Ethanolic extraction weight recovery for these products was between 26 and 55%.

From Table 1, it can be seen that CYP1A2, 2D6, and 3A4 were inhibited by all six herbs, but to different extents, with IC_{50} constants varying almost over a 200-fold range, from 23 ± 2 to 4323 ± 356 µg/ml. Only one herbal extract, cranberry, has similar IC_{50} constants for CYP1A2, 2D6, and 3A4 (95% CI). In contrast, both fennel and horsetail have different IC_{50} constants for all three CYP enzymes (95% CI). For CYP1A2, horsetail showed the strongest inhibition (IC_{50}: 27 ± 1 µg/ml), followed by raspberry leaf (IC_{50}: 44 ± 2 µg/ml) and fennel (IC_{50}: 115 ± 11 µg/ml). For CYP2D6, fennel had the lowest IC_{50} constant (23 ± 2 µg/ml), followed by raspberry leaf (47 ± 8 µg/ml) and horsetail (103 ± 0 µg/ml). For CYP3A4, the highest inhibition was by fennel (IC_{50}: 40 ± 4 µg/ml) and raspberry

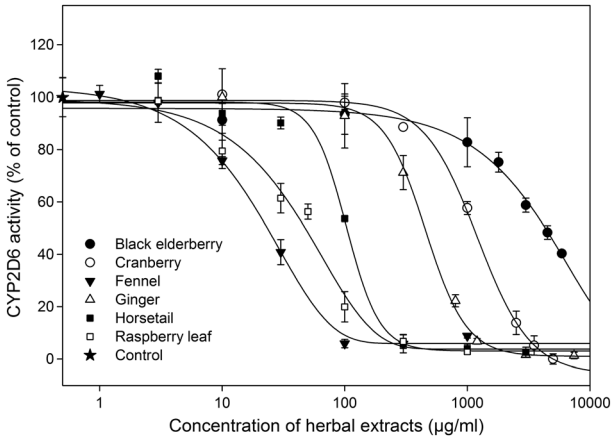

Figure 2. *In vitro* inhibition of CYP2D6 activity by the different herbal extracts. Experimental points represent mean values ± SD of triplicate incubations. The control samples without herbal extracts represent 100% enzyme activity. All curves fit the experimental points with $R^2 \geq 0.986$ ($p < 0.05$).

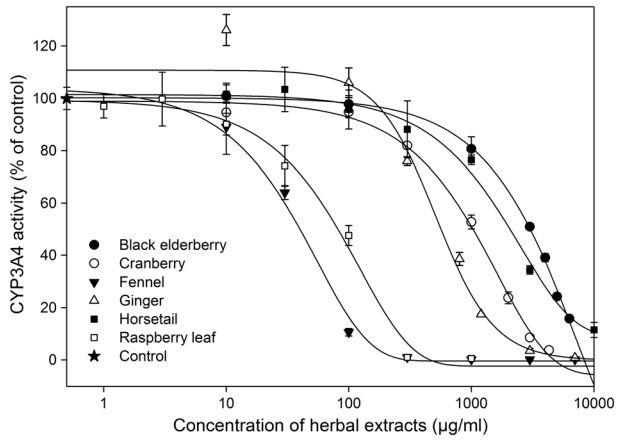

Figure 3. *In vitro* inhibition of CYP3A4 activity by the different herbal extracts. Experimental points represent mean values ± SD of triplicate incubations. The control samples without herbal extracts represent 100% enzyme activity. All curves fit the experimental points with $R^2 \geq 0.990$ ($p < 0.05$).

Table 1. *In vitro* inhibition of CYP activities by extracts obtained from commercially available herbal products frequently used in pregnancy and their recommended daily dosages

Herbal extract[a]	IC_{50} (μg/ml)[b]			Recommended daily dose
	CYP1A2	CYP2D6	CYP3A4	
Black elderberry	3612 ± 210	4323 ± 356	3086 ± 120	200 mg × 2
Cranberry	1228 ± 150	1132 ± 76	1056 ± 63	400 mg × 4
Fennel	115 ± 11	23 ± 2	40 ± 4	355 mg × 3
Ginger	320 ± 41	445 ± 35	565 ± 16	800 mg × 5
Horsetail	27 ± 1	103 ± 0	2064 ± 155	486 mg × 3
Raspberry leaf	44 ± 2	47 ± 8	81 ± 11	720 mg × 3

[a]Aqueous or 60% ethanolic extracts as described in Materials and methods.
[b]Mean IC_{50} constants ± SD, estimated from inhibition plots shown in Figures 1–3.

leaf (IC_{50}: 81 ± 11 μg/ml). The IC_{50} constants ± SD are summarized in Figure 4, showing the CYP inhibition profiles for all investigated herbs. The range of IC_{50} constants is equal for all three CYP enzymes ($p > 0.05$). Fennel and raspberry leaf are the overall strongest CYP inhibitors. The most diverse inhibition profile is seen for horsetail, having IC_{50} constants ranging from 27 ± 1 for CYP1A2, 103 ± 0 for CYP2D6, to 2064 ± 155 μg/ml for CYP3A4. Ginger, cranberry, and black elderberry have high IC_{50} constants for all the investigated CYP enzymes.

IC_{50}/IC_{25} CYP inhibition ratios, calculated from the inhibition plots in Figures 1–3, are shown in Table 2. In general, all herbs had lower IC_{50}/IC_{25} ratios for CYP1A2 than for CYP2D6 and 3A4, with mean ratios of 1.48 ± 0.04, 2.09 ± 0.44, and 2.21 ± 0.28, respectively ($p < 0.05$). No statistically significant difference in ratios was found between CYP2D6 and 3A4. The variation in ratios was also lower for CYP1A2 than for CYP2D6 and 3A4, with respective CVs of 3, 21, and 13.

DISCUSSION

Kimura *et al*. (2010) found that 100 μg/ml fennel inhibited CYP3A4 activity in human microsomes by approximately 80% (IC_{50} not specified). This is in accordance with the findings in the present study, where the same concentration would have inhibited this enzyme by 84%. Usia *et al*. (2006) observed less than 20% inhibition for both CYP2D6 and 3A4 in human microsomes when testing 1650 μg/ml of an aqueous fennel extract, while Subehan *et al*. (2006) found that a 500 μg/ml methanolic extract inhibited these enzymes by 15 and 31%, respectively. In comparison, these concentrations are far above those that fully inhibited both enzymes in this study. Such varying results could be due to unequal compositions of the active constituents in the crude herb or in the extract, for instance of bergapten (5-methoxypsoralen), a compound isolated from fennel, possessing a significant CYP3A4 inhibitory potential with an IC_{50} constant of 3.9 μg/ml (Subehan *et al*., 2007). Interestingly, bergapten is also an important constituent in grapefruit juice, which is well documented for inhibiting CYP3A4 *in vivo* (Ho *et al*., 2001).

No previous CYP interaction studies on raspberry leaf have been identified by us. It is indeed a frequently consumed herb in pregnancy (Nordeng *et al*., 2011) and should be closely investigated, especially as the ethanolic extract from raspberry leaf was one of the most potent CYP inhibitors in this study and thus strengthens the need for further documentation.

For horsetail, Sevior *et al*. (2010) found a low IC_{50} constant (13 μg/ml) for CYP1A2 and IC_{50} constants 100 μg/ml (not specified) for CYP2D6 and 3A4 in human liver microsomes. This applied for methanolic

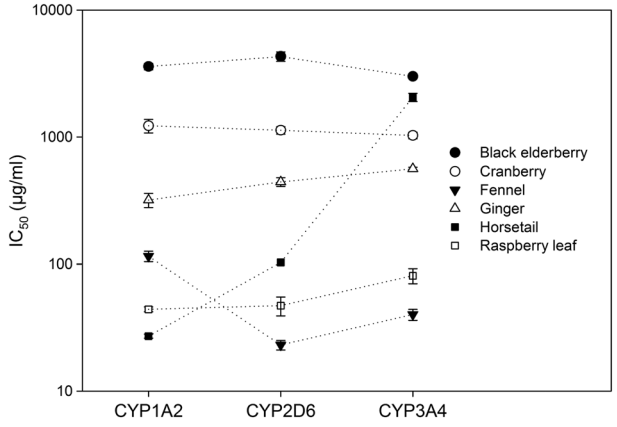

Figure 4. CYP inhibition profiles of the different herbal extracts. The points represent IC_{50} constants ± SD from triplicate incubations.

Table 2. IC_{50}/IC_{25} CYP inhibition ratios for extracts obtained from commercially available herbal products frequently used in pregnancy

Herbal extract[a]	IC_{50}/IC_{25} ratio[b]		
	CYP1A2	CYP2D6	CYP3A4
Black elderberry	1.46	2.68	2.17
Cranberry	1.43	1.85	2.37
Fennel	1.46	2.28	2.20
Ginger	1.52	1.63	1.67
Horsetail	1.46	1.66	2.41
Raspberry leaf	1.55	2.45	2.42

[a]Aqueous or 60% ethanolic extracts as described in Materials and methods.
[b]Estimated from inhibition plots shown in Figures 1–3.

extracts, while aqueous extracts had IC_{50} constants 100 μg/ml for all three enzymes. The first results indicate the same inhibitory pattern as found in this study, where the herb inhibits CYP1A2 to a much greater extent than the other two CYP enzymes. The aqueous extracts made in the present study involved boiling for 20 min, as this is how horsetail tea is prepared by consumers, and differ in this way from the aqueous extracts in the above-mentioned study. Scott *et al*. (2006) investigated the cDNA-expressed human CYP3A4 inhibitory potential of ethanolic horsetail extracts in two trials. The investigated concentrations were below our IC_{50} concentration, but the degree of inhibition was consistent with our results when extrapolating to the same concentrations within our CYP3A4 inhibition curve.

Reports on ginger's CYP inhibitory potential are inconsistent. Foster *et al*. (2003) reported that a 25 mg/ml aqueous ginger extract inhibited cDNA-expressed human CYP2D6 and 3A4 by 70 and 88%, respectively, and Kim *et al*. (2012) tested CYP inhibitory potentials of 5 μg/ml ethanolic ginger extracts with negligible effects on human microsome CYP1A2, 2D6, and 3A4 activities. In contrast, Kimura *et al*. (2010) found an IC_{50} constant as low as 5.1 μg/ml for CYP3A4 in human microsomes. In the present study, the IC_{50} constant was well above a 100-fold higher than that found by Kimura *et al*., but far below the high concentrations used by Foster *et al*. A possible explanation for such dispersed findings could be the large variance in composition of constituents for different ginger products, partially explained by numerous constituents that vary whether the ginger is fresh or dry, for instance volatile oil components and gingerols that degrades to shogaols (Schwertner *et al*., 2006). However, no conclusive results seem to exist at present.

Of relevant research on cranberry, Grenier *et al*. (2006) and Lilja *et al*. (2007) found, respectively, that a single or repeated daily ingestion of 240 or 200 ml cranberry juice did not inhibit *in vivo* human CYP activities significantly. This is in accordance with the *in vitro* findings of this study, where cranberry tablets have IC_{50} constants over 1000 μg/ml for the investigated enzymes. Ngo *et al*. (2009) found a considerable variation in inhibitory potential between different cranberry juice brands and suggest that such herbal products may alter intestinal, but not hepatic, human CYP3A.

Black elderberry is previously shown not to inhibit CYP3A4 significantly *in vitro* (Schroder-Aasen *et al*., 2012), which is consistent with our findings. No other studies are found for further comparison. The black elderberry product investigated in the present study also contained vitamin C, for which no CYP interactions are reported.

The IC_{50}/IC_{25} CYP inhibition ratios indicate how the degree of inhibition is affected by factorial changes in the herbal concentration, where a lower ratio implies a greater inhibition at dose enhancement. The lower and more uniform ratios found for CYP1A2 than for the two other enzymes thus indicate that herbal inhibition of CYP1A2 might be, although small, of higher toxicological significance than for CYP2D6 and 3A4. Furthermore, these findings indicate possible differences in CYP inhibition mechanisms for CYP1A2 as compared to CYP2D6 and 3A4, and further investigations should be performed to clarify this. The CYP3A4 IC_{50}/IC_{25} ratios in the present study are in the same range as previously reported for other herbs (Hellum and Nilsen, 2008; Hellum *et al*., 2010; Schroder-Aasen *et al*., 2012).

Findings presented in this study must be interpreted with caution regarding clinical relevance as results from *in vitro* studies with recombinant enzymes are not always consistent with the *in vivo* situation (Pelkonen *et al*., 2005). Pharmacokinetic properties of the herbal constituents, as bioavailability, distribution, and clearance, are important for estimation of the plasma concentration obtained from a given dose of an herbal product. Such pharmacokinetic parameters are, in contrast to marketed drugs, seldom known for herbal products. However, Strandell *et al*. (2004) suggest that if a recommended single herbal dose reaches its IC_{50} concentration when dissolved in a volume corresponding to the blood volume, its *in vivo* CYP inhibitory potential should be further investigated. For estimation of intestinal concentrations, Fenner *et al*. (2009) suggest a dissolution of an oral dose in a volume of 250 ml, while others use more than twice this volume (Hellum *et al*., 2007).

CYP1A2 is constitutively expressed in liver and is claimed not to contribute to the intestinal metabolism of orally administered xenobiotics (Zanger and Schwab, 2013). Estimates of herbal concentrations in the intestines are therefore not relevant for inhibition of this enzyme, but an inhibitory herbal plasma concentration equal to or above its IC_{50} concentration could affect *in vivo* metabolism by inhibiting hepatic CYP1A2. Horsetail and raspberry leaf showed the most potent inhibition towards CYP1A2. Their respective recommended single dosages of 486 and 720 mg divided by a blood volume of approximately 5 l gives concentrations of 97 and 144 mg/l, exceeding their respective IC_{50} concentrations of 27 and 44 mg/l by 3.6 and 3.3 times. For the remaining herbs, fennel, ginger, and especially cranberry and black elderberry, the IC_{50} constants are higher and plasma concentrations clinically relevant for CYP1A2 inhibition seem unlikely.

CYP2D6 is present both in the small intestine and in liver. Despite its relatively minor expression, there are a great number of drugs primarily metabolized by this enzyme (Zanger and Schwab, 2013). Estimates of possible achieved herbal concentrations in the small intestine, based on a recommended single dosage dissolved in an intestinal volume of 250 ml, show that both fennel and raspberry leaf exceed their IC_{50} constants over 60 times. One dose of horsetail, ginger, or cranberry gives respective concentrations of 19, 7, and 1 times their IC_{50} concentration, but for black elderberry, one dose will not reach its IC_{50} concentration in this volume. However, it should be noted that the clinical significance of a CYP2D6 inhibition in the intestine is questionable (Thelen and Dressman, 2009). As for CYP1A2, the two herbs showing the most potent inhibition towards CYP2D6, fennel and raspberry leaf, exceed their IC_{50} concentrations by just about a threefold when dividing their recommended single dosages by 5 l. For the remaining herbs, black elderberry, cranberry, ginger, and horsetail, *in vivo* inhibition of hepatic CYP2D6 is not likely.

CYP3A4 is the most abundantly expressed CYP enzyme in the intestines, and it is essential for intestinal metabolism (Zanger and Schwab, 2013). Inhibition of intestinal CYP3A4 has been shown to cause a clinically

significant increase in drug plasma concentration of drugs like, for instance, felodipine (Lown *et al*., 1997) and midazolam (Gorski *et al*., 1998). As for CYP2D6, fennel and raspberry leaf were the most potent *in vitro* inhibitors of CYP3A4. Single dosages of these herbs exceed their CYP2D6 IC_{50} concentrations just over 35 times in an intestinal volume of 250 ml. Ginger exceeds its IC_{50} concentration almost six times, and cranberry would reach its IC_{50} concentration, while the other herbs, horsetail and black elderberry, will not reach their respective IC_{50} concentrations in an equivalent volume. CYP3A4 is also generally highly expressed in liver, but with great inter-individual variability. Divided by a blood volume of approximately 5 l, the recommended single dosages for fennel and raspberry leaf exceed their IC_{50} concentrations by 1.8 times. None of the remaining herbs, black elderberry, cranberry, ginger, and horsetail, can reach such concentrations and inhibition of hepatic CYP3A4 seems unlikely.

In conclusion, as fennel, horsetail, and raspberry leaf today are frequently used in pregnancy, physicians should be aware of their potential for specific herb–drug interactions and recommend that these herbs should be used with care until further *in vivo* data are available.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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